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The Operation of the γ-Aminobutyrate Bypath of the Tricarboxylic Acid Cycle in Brain Tissue *in vitro*

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1. Cerebral-cortex slices prelabelled with γ-amino[1-^{14}C]butyrate (GABA) were incubated in a glucose–saline medium. After the initial rapid uptake there was no appreciable re-entry of ^{14}C into the GABA pool, either from the medium or from labelled metabolites formed in the tissue. The kinetic constants of GABA metabolism were determined by computer simulation of the experimental results by using mathematical procedures. The GABA flux was estimated to be 0.03 μmol per min/g, or about 8% of the total flux through the tricarboxylic acid cycle. It was found that the assumption of compartmentation did not greatly affect the estimates of the GABA flux. 2. The time-course of incorporation of ^{14}C into amino acids associated with the tricarboxylic acid cycle was followed with [1-^{14}C]GABA and [U-^{14}C]-glucose as labelled substrates. The results were consistent with the utilization of GABA via succinate. This was confirmed by determining the position of ^{14}C in the carbon skeletons of aspartate and glutamate formed after the oxidation of [1-^{14}C]GABA. These results also indicated that under the experimental conditions the reversal of reactions catalysed by α-oxoglutarate dehydrogenase and glutamate decarboxylase respectively was negligible. The conversion of [^{14}C]GABA into γ-hydroxybutyrate was probably also of minor importance, but decarboxylation of oxaloacetate did occur at a relatively slow rate. 3. When [1-^{14}C]GABA was the labelled substrate there was evidence of a metabolic compartmentation of glutamate since, even before the peak of the incorporation of ^{14}C into glutamate had been reached, the glutamine/glutamate specific-radioactivity ratio was greater than unity. When [U-^{14}C]glucose was oxidized this ratio was less than unity. The heterogeneity of the glutamate pool was indicated also by the relatively high specific radioactivity of GABA, which was comparable with that of aspartate during the whole incubation time (40 min). The rates of equilibration of labelled amino acids between slice and medium gave evidence that the permeability properties of the glutamate compartments labelled as a result of oxidation of [1-^{14}C]GABA were different from those labelled by the metabolism of [^{14}C]glucose. The results showed therefore that in brain tissue incubated under the conditions used, the organization underlying metabolic compartmentation was preserved. The observed concentration ratios of amino acids between tissue and medium were also similar to those obtaining *in vivo*. These ratios decreased in the order: GABA > acidic acids > neutral amino acids > glutamine. 4. The approximate pool sizes of the amino acids in the different metabolic compartments were calculated. The glutamate content of the pool responsible for most of the labelling of glutamine during oxidation of [1-^{14}C]GABA was estimated to be not more than 30% of the total tissue glutamate. The GABA content of the 'transmitter pool' was estimated to be 25–30% of the total GABA in the tissue. The structural correlates of metabolic compartmentation were considered.

Under normal conditions glucose is the main substrate utilized by the brain (Balázs, 1969). The metabolism of glucose proceeds in the brain, as in other organs, mainly by glycolysis and oxidation of pyruvate in the tricarboxylic acid cycle. However, the brain differs from other organs in having an

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alternative route of oxidation through α-oxoglutarate, the so-called GABA* bypath (Scheme 1; Elliott, 1965; Roberts & Kuriyama, 1968).

(1) α-Oxoglutarate + GABA $\underset{\text{(GABA aminotransferase)}}{\rightleftharpoons}$ glutamate + succinic semialdehyde

(2) Succinic semialdehyde + NAD^+ $\xrightarrow[\text{dehydrogenase)}]{\text{(succinic semialdehyde}}$ succinate + NADH + H^+

(3) Glutamate $\xrightarrow[\text{decarboxylase)}]{\text{(glutamate}}$ GABA + CO_2

Sum: α-Oxoglutarate + NAD^+ ⟶ succinate + CO_2 + NADH + H^+

The operation of the GABA bypath is indicated by the occurrence of an active glutamate decarboxylase in the brain and by the evidence that glucose carbon is converted into GABA in the brain (cf. Beloff-Chain, Catanzaro, Chain, Masi & Pocchiari, 1955; Cremer, 1964; Gaitonde, 1965). It has been shown that [^{14}C]GABA is metabolized in brain tissue both *in vivo* and *in vitro* (cf. Tsukada, Hirano, Nagata & Matsutani, 1960; Roberts & Kuriyama, 1968). However, the quantitative contribution of the GABA bypath to glucose oxidation in the brain is not yet known; estimates vary from about 10 to 40% of the total flux through the tricarboxylic acid cycle (Haslam & Krebs, 1963; Balázs & Haslam, 1965; Elliott, Khan, Bilodeau & Lovell, 1965; Haber, 1965; Van Gelder, 1966; Garfinkel, 1966; McKhann, Albers, Sokoloff, Mickelsen & Tower, 1960).

The uncertainty in the estimation of the GABA flux stems mainly from the compartmentation of glutamate in the brain (Albers, Koval, McKhann & Ricks, 1961; Garfinkel, 1966). In the present investigation the metabolism of GABA was studied in slices of guinea-pig cerebral cortex and an attempt was made to avoid the difficulties resulting from the compartmentation of glutamate by prelabelling the GABA pools of the tissue with [1-^{14}C]GABA. When GABA is metabolized C-1 is quickly lost as carbon dioxide and it could be shown that under the experimental conditions used there was no significant re-entry of ^{14}C into the GABA pools. The fractional utilization rate of GABA could therefore be calculated from the rate of decay of [1-^{14}C]GABA in slices metabolizing glucose. The mathematical approach is described in the Appendix (Hammond, Julian, Machiyama & Balázs, 1970). By observing the rate of entry of ^{14}C from GABA into other metabolites a comprehensive picture of the metabolism of GABA was obtained.

* Abbreviation: GABA, γ-aminobutyrate.

Metabolic compartmentation of glutamate in the brain during the utilization of [^{14}C]GABA has been observed both *in vitro* (Balázs, Machiyama & Richter, 1967) and *in vivo* (Baxter, 1968). Compartmentation of the tricarboxylic acid cycle was also suggested by studies of the metabolism of various different labelled substrates in the brain (Berl & Clarke, 1969; Van den Berg, 1970) and a model was put forward by Garfinkel (1966) on the basis of computer simulation of experimental data. Investigations have given evidence that GABA is also compartmented in cerebral-cortex slices (Machiyama, Balázs & Richter, 1967; Machiyama, Balázs, Hammond, Julian & Richter, 1970). The nature of metabolic compartmentation was therefore studied by comparing the metabolic pattern resulting from the utilization of two different precursors, one of which ([1-^{14}C]GABA) appears to be compartmented, whereas the other ([^{14}C]-glucose) does not.

Part of this investigation has been reported in a preliminary form (Machiyama, 1965; Machiyama, Balázs & Julian, 1965; Balázs *et al.* 1967).

EXPERIMENTAL

Experimental animals and preparation of slices. Adult male guinea pigs from the same colony, weighing 375–425g, and albino rats of the Porton strain were used. Slices of cerebral cortex, 0.3–0.35mm in thickness, were cut at room temperature in a humid chamber gassed with O_2 as described by Elliott (1955). We are indebted to Professor Elliott for his help in setting up this method. Each hemisphere yielded three slices: the first and second slices from the fronto-parietal region and the first slice from the temporo-occipital region. To decrease variability the slices were always prepared from identical areas. The weight of the six slices obtained from one brain was adjusted to a total of 200mg by cutting off the excess from one of the second slices from the fronto-parietal area. Each incubation for one time-point in the time-course study was performed with slices from one brain.

Incubation and manometric techniques. The incubation of slices started at 25min after the animals had been killed. It was observed that the increase in GABA content slowed down considerably at about 20min after death; thus it was possible to obtain relatively constant values of GABA at the beginning of the incubation.

The glucose–saline medium was oxygenated for 10min before use and had the following composition: 127mM-NaCl, 4.7mM-KCl, 2.5mM-$CaCl_2$, 1.2mM-$MgSO_4$, 1.2mM-KH_2PO_4, 30mM-tris–HCl buffer, pH7.4, and 10mM-glucose. Unless otherwise stated the procedure was as follows. Slices were placed in the main compartment of Warburg vessels containing 2.9ml of the glucose–saline medium. The labelled substrates, 0.5μCi of [1-^{14}C]GABA or 5μCi of [U-^{14}C]glucose, were placed in the side arm; folded filter paper and 0.2ml of 2M-KCl were in the centre well. After 5min the vessels were placed in a bath at 37.5°C and gassed for 5min with O_2. The labelled substrate

was added at 10min after the start of the incubation (final volume 3ml). O_2 uptake was measured every 5min.

After incubation, slices were separated from the medium by filtration through a porcelain disc covered with Whatman no. 50 paper and immediately fixed in ice-cold 0.5M-$HClO_4$. To keep the time of these operations as short as possible (1.5–2min) the slices were not washed. Correction for the medium contaminating the slices was not necessary, since the concentration of amino acids in the medium compared to that in the slices was very small and the maximum contamination of the tissue by medium was about 0.15ml. A part of the clear supernatant (2.5ml) obtained from the separated medium by centrifugation was acidified with 0.2ml of 9.2M-$HClO_4$.

When $^{14}CO_2$ was determined the reactions were stopped by tipping 0.3ml of 9.2M-$HClO_4$ from the second side arm.

Isolation of amino acids by chromatographic procedures. Slices were homogenized in 0.5M-$HClO_4$ with a Teflon pestle and the suspension as well as the acidified incubation medium was centrifuged. The residues were washed with 2×1ml of 0.4M-$HClO_4$ and the supernatants combined. The extracts were neutralized with KOH. The precipitate was removed by centrifugation and was washed with 2×1ml of ice-cold water. The neutralized extract was fractionated by the method of Gaitonde (1965). An advantage of this method is that the amount and ^{14}C content of amino acids are determined after elution from unstained paper; thus a loss of ^{14}C due to the ninhydrin reaction is avoided. In principle the method allows the separation of carboxylic acids and neutral compounds (fraction I) from amino acids (fraction II). Fraction II is then separated by ion-exchange chromatography into basic, neutral and acidic amino acids. Fraction I when further analysed was processed as described by Gaitonde, Marchi & Richter (1964) and yielded three fractions: (*a*) 'glucose' fraction (glucose and other reducing compounds), (*b*) taurine and other unknown substances, and (*c*) 'carboxylic acid' fraction.

The amino acids were separated by paper chromatography (Whatman no. 1 paper). The solvent system for the neutral and acidic amino acid fractions was butan-1-ol–propionic acid–water (10:5:7 and 10:5:4, by vol., respectively). The amino acids were located by scanning the paper strips with a BTL chromatogram counter and also by comparison with marker strips run at the same time on both sides of the sample. Under these conditions serine, glycine and glutamine were not well separated. The area corresponding to these amino acids was therefore eluted; rechromatography with 85% (w/v) phenol resulted in good separation.

The 'glucose' fraction was further resolved by paper chromatography by the method of Gaitonde *et al.* (1964).

Recoveries were checked by determining the radioactivity at each step of the separation procedures and were found to be 90–105%, except for the acidic amino acid fraction from the medium, for which it was approx. 80%.

Enzymic decarboxylation of glutamate and aspartate. To determine the position of the ^{14}C in glutamate and aspartate after the incubation with [1-^{14}C]GABA, C-1 of glutamate and C-4 of aspartate were removed by decarboxylation with *Clostridium welchii* and *Nocardia globerula* preparations respectively (Haslam & Krebs, 1963). We are grateful to Mr A. Tuffrey (M.R.C. Laboratory Animal Centre, Carshalton, Surrey, U.K.) for supplying these preparations. The conditions for the incubation and the counting of the radioactivity of the $^{14}CO_2$ were as described by Balázs (1965).

Analytical techniques. Amino N was determined by the method of Yemm & Cocking (1955). The samples were prepared as described by Gaitonde, Dahl & Elliott (1965). Amounts of amino acids were also determined on chromatographed paper strips used as markers to locate the unstained strips, by the method of Gaitonde (1961). Most of the amino acids in the incubation medium, which were present in small quantities, could be measured only by this method. Glucose separated by paper chromatography was determined by the method of Park & Johnson (1949).

Radioactive-tracer techniques. The radioactivity of the samples (except $^{14}CO_2$) was counted on plastic planchets with a Nuclear–Chicago gas-flow counter. Correction for self-absorption, background and dead time were applied. Respiratory $^{14}CO_2$ was converted into $Ba^{14}CO_3$ and was counted as described by Balázs (1965).

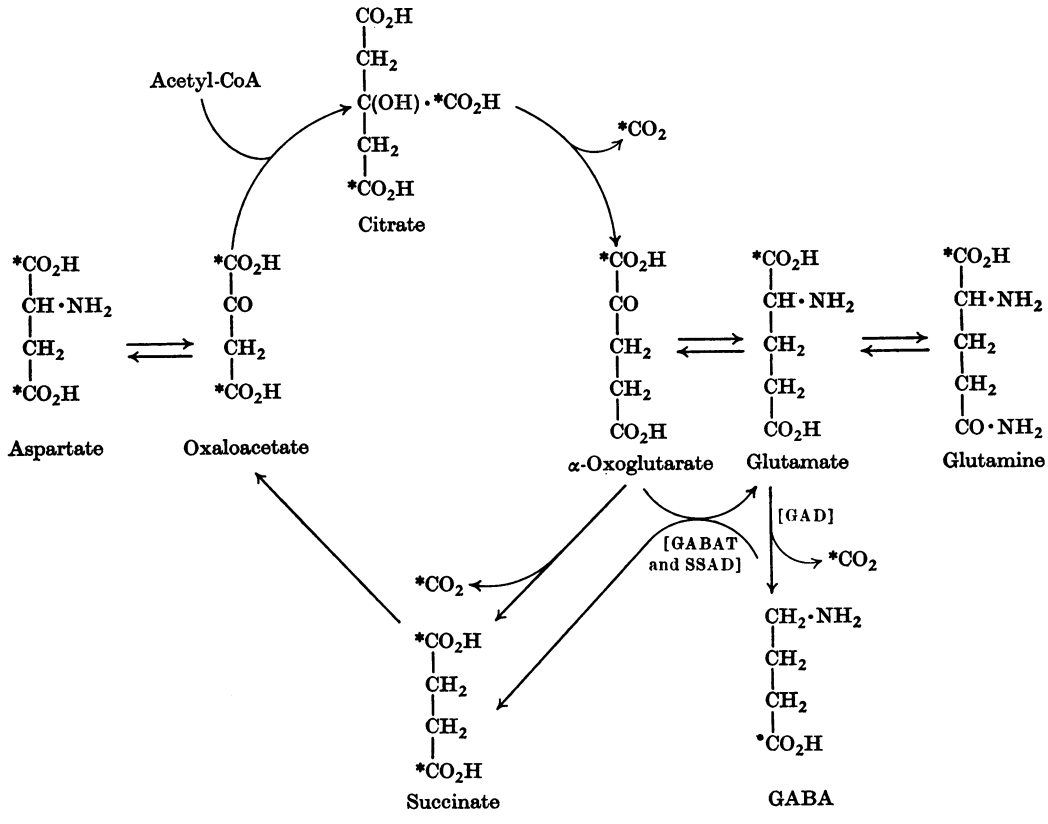
Chemicals. D-[U-^{14}C]Glucose (76.3mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and [1-^{14}C]GABA (3.72mCi/mmol) from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

All reagents were of analytical grade. Glass-distilled water was used for the preparation of the incubation medium, and for other purposes deionized water passed through an Elgastat deionizer was used.

RESULTS

Uptake of [1-^{14}C]*GABA from the medium.* [1-^{14}C]GABA was taken up rapidly by slices of guinea-pig cerebral cortex incubated in glucose–saline medium. At 10min after the addition of 0.135μmol of [1-^{14}C]GABA (108000c.p.m.), 95% of the ^{14}C had already been taken up by the tissue; and during further incubation the amount of [^{14}C]GABA in the medium was only 3% of that in the slices (Tables 1 and 2). GABA was well retained by the tissue during the whole incubation time (110min), and the amount found in the medium was less than the resolution of the method used for amino acid determination. Thus under the present experimental conditions, the GABA pool in the tissue could be labelled by externally added [^{14}C]GABA, and after a short initial period both the uptake of GABA and the release of GABA from the tissue could be neglected.

Re-entry of ^{14}C *into the GABA pool in the tissue.* To ensure that no ^{14}C re-entered the [^{14}C]GABA pool from labelled intermediates formed in the tissue, GABA labelled at C-1 was used. It is believed that GABA utilization proceeds through succinate in the tricarboxylic acid cycle (see Elliott, 1965; Roberts & Kuriyama, 1968). The fate of the labelled carbon according to that scheme is depicted in Scheme 1. [1-^{14}C]GABA is converted into [1-^{14}C]glutamate, whose labelled carbon atom is lost as $^{14}CO_2$ when GABA is re-formed by



Scheme 1. Abbreviated scheme of metabolism of [1-^{14}C]GABA. The labelled substrate is [1-^{14}C]GABA (position of label is shown by a solid circle). The position of the labelled carbon in the metabolites formed when [1-^{14}C]GABA is utilized through succinate in the tricarboxylic acid cycle is indicated with an asterisk. The enzymes related to GABA metabolism are given in square brackets: GAD, glutamate decarboxylase; GABAT, GABA aminotransferase; SSAD, succinic semialdehyde dehydrogenase.

decarboxylation. This was confirmed experimentally: glutamate was isolated from the slices after incubation in a medium containing [1-^{14}C]GABA and the amount of ^{14}C was determined in C-1 of glutamate after decarboxylation with *Cl. welchii* (Table 3). About 98% of the recovered ^{14}C was found at C-1 of glutamate. The same results were obtained when the respiration rate was stimulated by the addition of 60mM-potassium chloride. Therefore, after the initial uptake of [^{14}C]GABA by the slice, no significant re-entry of ^{14}C into the labelled GABA pool can be expected, either from the medium or from tissue metabolites, under the experimental conditions used.

Effect of GABA on respiration rate. The respiration rate of guinea-pig cerebral-cortex slices was constant under our experimental conditions for almost 90min of incubation [72.8±0.25 (9) μmol of oxygen/h per g fresh tissue] and there was only a slight decrease in the oxygen uptake (3–5%) when the incubation was further prolonged. The effect of GABA on the oxygen consumption of cerebral-cortex slices incubated in the glucose–saline was investigated and the respiration rate was found to be increased by 10 and 30% respectively in the presence of 1mM- or 10mM-GABA. The oxygen uptake was apparently unaffected by 0.1mM-GABA. The initial concentration of GABA in the following experiments was only 0.04mM.

Metabolism of [1-^{14}C]*GABA.* The utilization of [^{14}C]GABA was followed by determining the ^{14}C content and distribution in the acid-soluble fraction of the tissue and in the medium at 10min intervals up to 110 min during incubation in the glucose–saline medium. The acid-insoluble fraction was not analysed, since preliminary experiments showed that the ^{14}C content of this fraction was relatively low. Analysis of the results indicated that the total ^{14}C was satisfactorily accounted for by the radioactivity recovered in the fractions studied (recoveries

Table 1. *Distribution of* ^{14}C *in the acid-soluble fraction of guinea-pig cerebral-cortex slices and medium during incubation in glucose–saline medium containing* [1-^{14}C]*GABA*

Unmoistened guinea-pig cerebral-cortex slices (0.3–0.35mm thick) were prepared in a humid chamber gassed with O_2 (Elliott, 1955). The slices (200mg wet wt.) were put into Warburg vessels containing glucose–saline medium (see the Experimental section) at 25min after the animals were killed. At 30min the vessels were placed in the water bath (37.5°C) and were gassed with O_2. [1-^{14}C]GABA (108000c.p.m.) was tipped from the side arm at 10min after the beginning of the incubation (zero time), and slices and medium were separated at the times indicated and analysed as described in the Experimental section. The ^{14}C contents in the acid-soluble fractions of slice and medium were determined directly and the recovery of ^{14}C after fractionation is indicated.

Time after addition of [1-^{14}C]GABA (min) ...	10	20	30	40	50	60	70	80	90	100	110
Slice											
^{14}C determined in acid-soluble subfractions ($10^{-3}\times$c.p.m.)	87.5	78.8	70.0	60.8	49.6	41.8	37.3	29.3	27.6	25.5	25.3
Recovery of ^{14}C in acid-soluble subfractions (%)	100	100	98	103	104	106	107	104	101	104	106
^{14}C in acid-soluble subfractions as percentage of recovered ^{14}C:											
Carboxylic acid fraction	0.93	1.19	1.43	1.80	2.11	2.32	2.69	3.44	3.48	3.69	3.84
Basic amino acid fraction	2.63	2.56	2.34	2.41	3.04	3.33	3.75	4.23	3.79	4.50	4.53
Neutral amino acid fraction	88.1	84.8	81.6	78.6	74.7	72.8	75.1	74.3	74.4	72.2	75.1
Acidic amino acid fraction	8.34	11.5	14.6	17.2	20.2	21.6	18.4	18.1	18.4	19.6	16.5
Medium											
^{14}C recovered in acid-soluble subfractions ($10^{-3}\times$c.p.m.)	8.23	5.39	5.47	6.02	7.29	7.51	9.04	8.36	8.89	9.04	8.27
Recovery of ^{14}C in acid-soluble subfractions (%)	94	94	95	94	96	96	100	94	99	94	92
^{14}C in acid-soluble subfractions as percentage of recovered ^{14}C:											
Carboxylic acid fraction	11.5	26.0	34.5	37.6	34.7	39.0	35.9	37.7	37.1	34.4	38.2
Basic amino acids	6.2	7.1	7.9	6.4	3.3	3.1	2.6	2.4	2.4	2.5	3.4
Neutral amino acids	75.9	55.8	46.5	44.9	52.4	48.5	54.5	52.1	53.7	55.1	49.8
Acidic amino acids	6.4	11.0	11.0	11.0	9.6	9.3	6.9	7.7	6.8	7.9	8.6

Table 2. *Time-course of* [1-^{14}C]*GABA utilization by slices of guinea-pig cerebral cortex incubated in glucose–saline medium containing* [1-^{14}C]*GABA*

The results are derived from the experiments given in Table 1. Values are expressed (*A*) as ^{14}C radioactivity (c.p.m.) and (*B*) as percentage of [^{14}C]GABA utilized.

Time after addition of [1-^{14}C]GABA (min)...	10		20		30		40		50		60		70		80		90		100		110	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
[^{14}C]GABA recovered:																						
in slice	76 909		66 274		52 718		43 573		35 818		25 991		25 991		16 626		15 809		15 373		16 209	
in medium	5327		1851		1408		1139		982		710		857		551		498		661		576	
[^{14}C]GABA utilized*	25 764	(100)	39 875	(100)	53 874	(100)	63 288	(100)	71 200	(100)	81299	(100)	81152	(100)	90823	(100)	91693	(100)	91966	(100)	91215	(100)
[^{14}C]GABA converted into amino acids:																						
in slice	9767	38	11504	29	16274	30	16106	25	12719	18	14793	18	10300	13	11707	13	10782	12	9152	10	8103	8.9
in medium	1958	7.6	2133	5.4	2177	4.0	2617	4.1	3780	5.3	3863	4.8	4937	6.1	4656	5.1	5098	5.6	5270	5.7	4534	5.0
in slice plus medium	11725	46	13727	34	18451	34	18723	30	16499	23	18656	23	15237	19	16363	18	15880	17	14422	16	12637	14
[^{14}C]GABA converted into neutral compounds and carboxylic acids:																						
in slice	818	3.2	939	2.4	1000	1.9	1091	1.7	1048	1.5	970	1.2	1004	1.2	1010	1.0	960	1.1	939	1.0	970	1.1
in medium	944	3.7	1403	3.5	1888	3.5	2266	3.6	2527	3.6	2934	3.6	3245	4.0	3155	3.5	3298	3.6	3110	3.4	3163	3.5
in slice plus medium	1762	6.9	2342	5.9	2888	5.4	3357	5.3	3575	5.0	3904	4.8	4249	5.2	4165	4.6	4258	4.6	4049	4.4	4133	4.5
[^{14}C]GABA converted into $^{14}CO_2$†	12 277	48	23 806	60	32 535	60	41 208	65	51 126	72	58 739	72	61 666	76	70 295	77	71555	78	73495	80	74445	82

* [^{14}C]GABA utilized = [^{14}C]GABA added (108 000c.p.m.) − [^{14}C]GABA recovered at times indicated.

† Calculated value: [^{14}C]GABA utilized − ^{14}C recovered in acid-soluble fraction in compounds other than GABA. In one experiment $^{14}CO_2$ production was estimated at 1h after the addition of [1-^{14}C]GABA and the measured value was near to that calculated in the Table.

94–107%; Table 1). The ^{14}C recovered in the tissue amounted to nearly 90% of the ^{14}C in the acid-soluble fraction of slices plus medium at the beginning of the incubation and to more than 70% at the end (Table 1). A high proportion of the ^{14}C was combined in amino acids; the fraction containing the basic amino acids contained only small amounts of ^{14}C, and this fraction was therefore not analysed further.

The amount of [1-^{14}C]GABA utilized could be calculated from the results in Tables 1 and 2. The results show that approx. 90% of the added [^{14}C]GABA was metabolized in 110 min (Table 2). At 10min almost 50% of the metabolized [1-^{14}C]-GABA was converted into other amino acids. A large proportion of the GABA carbon was continuously lost from the system, probably as $^{14}CO_2$, which at 110min amounted to about 70% of the [^{14}C]GABA added.

The maximum ^{14}C content in the amino acids in the medium occurred at about 1.5h after addition of [^{14}C]GABA, whereas in the tissue the peak was reached at 0.5h. These observations are related to the differences in the composition of [^{14}C]amino acids in the tissue and medium (see Fig. 3). Only a small proportion of the ^{14}C in the slice was in the fraction containing the carboxylic acids whereas in the medium this fraction contained 40% of the total ^{14}C. However, even in the medium the ^{14}C combined in carboxylic acids at any time was less than 4% of the total [^{14}C]GABA utilized.

It was observed that the time-course of the labelling of the amino acids and the carboxylic acids in the tissue was different from that in the medium. Therefore, to avoid possible artifacts due to the introduction of 'experimental' compartmentation, the tissue and the medium had to be analysed separately. This possibility was not excluded in the experiments of Simon, Drori & Cohen (1967), whose analyses were carried out with pooled material from tissue and medium. These authors observed that the specific radioactivities of some tricarboxylic acid cycle intermediates were lower than those of the corresponding amino acids and concluded that their findings implied metabolic compartmentation in the tissue.

Pathway of GABA metabolism. The ^{14}C combined in GABA decreased during the incubation, indicating that GABA was utilized (Table 2). The progressive decrease in specific radioactivity of GABA showed that GABA was also re-formed from glutamate (Fig. 1).

The sequence of appearance of ^{14}C from [^{14}C]-GABA in the amino acids associated with the tricarboxylic acid cycle was consistent with the pathway given in Scheme 1. ^{14}C appeared at first in aspartate, whose peak of specific radioactivity was reached at 20–30min. The considerable difference

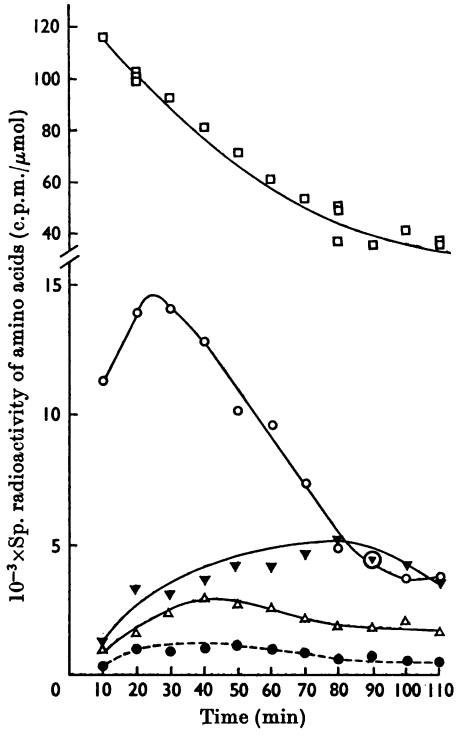


Fig. 1. Time-course of conversion of [1-^{14}C]GABA into amino acids in slices of guinea-pig cerebral cortex incubated in glucose–saline medium. The experimental conditions were described in Tables 1 and 2. The specific radioactivities of the amino acids are expressed as c.p.m./μmol. □, GABA; ○, aspartate; △, glutamate; ▼, glutamine; ●, alanine.

between the specific radioactivities of asparate and GABA at that time can be attributed to the flow of unlabelled carbon atoms into the aspartate pool as a result of the operation of the tricarboxylic acid cycle. The results indicate that under the experimental conditions only a fraction of glucose utilization was channelled through the GABA bypath.

The amino acid that showed a peak in incorporation of ^{14}C immediately after that of aspartate was glutamate (at 40min). To compare the specific radioactivities of aspartate and glutamate, the value for glutamate must be multiplied by 2, since one labelled carbon atom is lost when isocitrate formed from [1,4-^{14}C]oxaloacetate is decarboxylated to α-oxoglutarate (see Scheme 1). However, the specific radioactivity of aspartate at 40min was still about twice that of glutamate. One explanation of these results is that only a part of the total amount of glutamate is associated with the flow of carbon atoms from the labelled aspartate pool. Another observation indicating that glutamate is compartmented in respiring cerebral-cortex slices was the finding that the specific radioactivity of glutamine (maximum at 80min) was higher throughout the whole incubation time than that of glutamate, which is the precursor of glutamine (Fig. 1).

Incorporation of ^{14}C into alanine was observed. This can result from the decarboxylation of labelled C_4 dicarboxylic acids. The specific radioactivity of alanine was low in comparison with that of aspartate during the whole incubation; the peak was reached at 50min after the addition of [1-^{14}C]GABA and the specific radioactivity at that time was about 20% of that of aspartate (the specific radioactivity of alanine for comparison with that of aspartate must be multiplied by 2, since one ^{14}C atom is lost when pyruvate is formed from [^{14}C]dicarboxylic acids). Since about 50% of the ^{14}C combined in aspartate was in the C-4 position (Table 3), carbon dioxide fixation was probably of minor significance under the experimental conditions used (continuous absorption of carbon dioxide in alkali).

The ^{14}C content of serine and glycine was very low. In the present experiments no precautions were taken to preserve GSH during the processing of the samples, but the region of the chromatograms corresponding to this peptide and its derivatives did not contain appreciable ^{14}C.

The ^{14}C content of the fraction containing neutral compounds and carboxylic acids was very low during the whole incubation (Tables 1 and 2). These results suggest that the reduction of succinic semialdehyde to γ-hydroxybutyrate (Fishbein & Bessman, 1964) was of minor importance under the conditions used.

The observations described in this section indicated that [^{14}C]GABA was metabolized in brain-cortex slices via succinate in the tricarboxylic acid cycle. Further evidence was provided by the determination of the position of ^{14}C in aspartate and glutamate, which were isolated from the tissue after incubation with [1-^{14}C]GABA. The carbon atoms at C-4 of aspartate and C-1 of glutamate were removed as carbon dioxide by treatment with *Nocardia globerula* and *Cl. welchii* respectively (Table 3). About 50% of the ^{14}C in aspartate was in C-4 and approx. 98% of the ^{14}C in glutamate was in C-1. The oxidation of [1-^{14}C]GABA according to Scheme 1 results in the formation of [1,4-^{14}C]oxaloacetate due to randomization of carbon in succinate; thus 50% of the ^{14}C in aspartate should be at C-4. Half of the ^{14}C in isocitrate derived from the labelled oxaloacetate is removed by decarboxylation when α-oxoglutarate is formed; the ^{14}C in glutamate is therefore in C-1.

The observation that ^{14}C in glutamate was almost exclusively in C-1 demonstrated that the reactions catalysed by glutamate decarboxylase and α-oxoglutarate dehydrogenase are irreversible under our experimental conditions. If these reactions were

Table 3. *Position of* ^{14}C *in aspartate and glutamate isolated from slices after incubation in glucose–saline medium containing* [1-^{14}C]*GABA*

Slices of guinea-pig cerebral cortex were incubated in glucose–saline medium containing [1-^{14}C]GABA (108000 c.p.m.) in the presence of the usual concentration (4.7 mM) and of high concentration (plus 60 mM) of KCl for 30–60 min. After the termination of incubation aspartate and glutamate were isolated from slices by the method given in the Experimental section. The amino acids were decarboxylated with *Nocardia globerula* and *Cl. welchii* respectively.

Amino acid	Expt. no.	KCl (mM)	^{14}C in amino acid (c.p.m.)	^{14}C removed by decarboxylation (c.p.m.)	^{14}C removed by decarboxylation (% of recovered ^{14}C)	Residual ^{14}C (c.p.m.)	Recovery (%)
Aspartate	1	4.7	10924	5284	51	5081	95
	2	4.7	3620	1956	53	1767	103
	3	64.7	4198	2005	50	1988	95
Glutamate	1	4.7	9439	8995	98	187	97
	2	4.7	4749	4567	97	140	99
	3	64.7	7228	6738	97	203	96

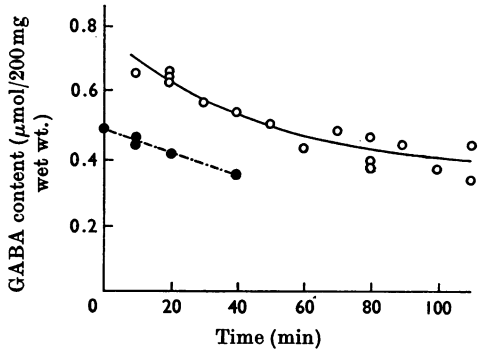


Fig. 2. Perturbation of GABA content in slices of guinea-pig cerebral cortex incubated (1) in [U-^{14}C]glucose–saline medium (●, data from the experiments given in Table 4), and (2) in glucose–saline medium that at zero time contained 0.135 μmol of [1-^{14}C]GABA (○, data from the experiments described in Tables 1 and 2). The slices had been preincubated in glucose–saline medium for 10 min before the labelled substrates were added (experimental zero time). The results were simulated by an analogue computer by assuming a homogeneous pool of GABA, giving a good fit (Fig. 1*a* of the Appendix); an equally good fit was obtained by assuming compartmentation of GABA (Fig. 1*c* of the Appendix). For details see the Appendix (Hammond *et al.* 1970).

reversible then appreciable radioactivity should also be contained in carbon atoms other than C-1 of glutamate.

Changes in the concentration of amino acids in slices. The GABA content of the slices at the beginning of the incubation (0.66 μmol) was much higher than the value *in vivo* (approx. 0.3 μmol/200 mg of tissue), which was obtained by extrapolating the post-mortem changes in GABA content. The high initial value of GABA was due partly to an increase in the amount present in brain after death (Elliott, 1965) and partly to the rapid uptake of GABA from the medium (compare the content of GABA in slices incubated with or without added GABA; Fig. 2). Computer analyses showed an exponential decrease in the content of GABA during incubation, approaching a steady-state value of 0.33 μmol/200 mg of tissue.

The amount of glutamate in the slices was fairly constant (about 10 μmol/g) up to 90 min but decreased later (about 7.5 μmol/g at 110 min); this was attended by a sharp increase of glutamine content in the medium. The aspartate content of the tissue was relatively constant for about 70 min (Fig. 6). The contents of glutamine and alanine in the tissue were relatively constant during the whole incubation time (about 3.5 and 1.5 μmol/g respectively). The amounts of glycine and particularly of serine increased throughout the incubation period (Table 5).

Amino acids in the incubation medium. After the initial uptake the amount of GABA in the medium was not measurable, but it could be estimated from the ^{14}C content (Fig. 3*b*) assuming that the specific radioactivity of GABA was the same as in the slice (Fig. 1): the calculated value of the concentration was 4–6 nM. The amounts of the other neutral amino acids studied could be determined in the medium (Tables 4 and 5). The major amino acid constituent of the medium was glutamine, which also contained a high proportion of the ^{14}C (Fig. 3*b*). In contrast with the tissue, in which the glutamine concentration was fairly constant, the glutamine concentration in the medium increased from about 0.2 to more than 0.4 mM during 110 min of incuba-

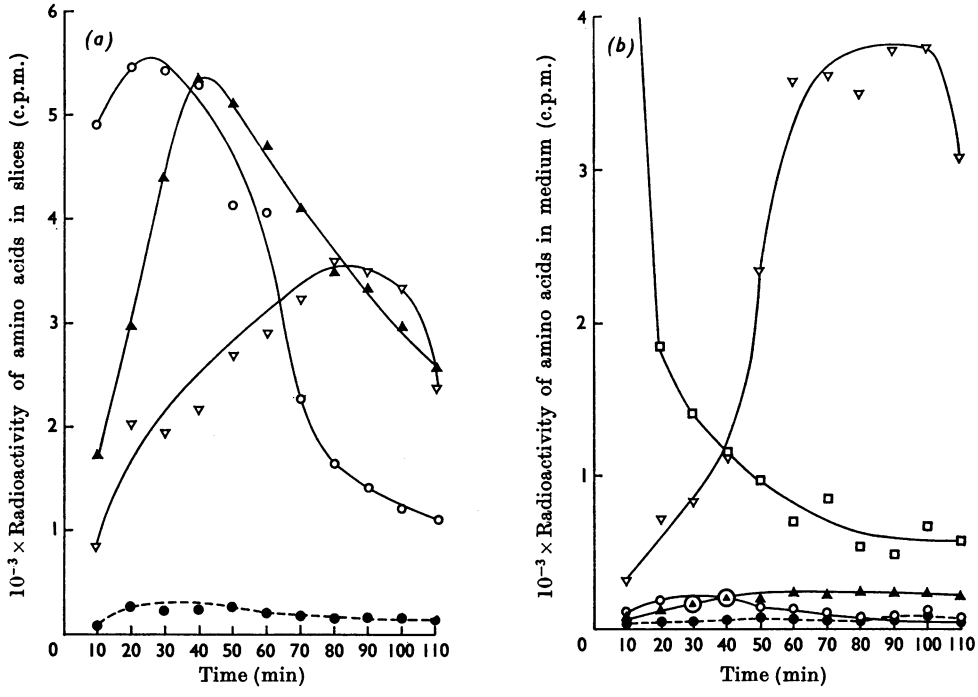


Fig. 3. Time-course of incorporation of ^{14}C in amino acids from [1-^{14}C]GABA in (*a*) slices of guinea-pig cerebral cortex (200mg fresh wt.) and (*b*) incubation medium (3ml). Experimental details were given in Tables 1 and 2. ○, Aspartate; ▲, glutamate; ▽, glutamine; ●, alanine; □, GABA (in medium only; for values of [^{14}C]GABA in slice see Table 2).

tion. A part of the increase of glutamine in the medium was apparently at the expense of the glutamate in the tissue. The total amount of glutamate in 200mg of tissue decreased by about 0.4μmol and that of glutamine increased by about 0.7μmol. The concentrations of the other neutral amino acids were fairly constant during the incubation. The amount of acidic amino acids that leaked out into the medium was relatively low, only about 10% of that found in the tissue, and it remained constant during incubation (concentrations of glutamate and aspartate, 40 and 10μM respectively).

It appears, therefore, that the concentrations of amino acids (except for glutamine) in the medium were fairly constant. However, the situation was not static, since the specific radioactivities changed in the medium apparently following those of the amino acids in the slice. Initially the specific radioactivities of the amino acids in the tissue were much higher than those in the medium and it took more than 1h until the values approached equilibrium (Fig. 4*a*). Glutamate was much nearer to equilibrium in slice and medium than were the other amino acids, even at short times after the addition of [^{14}C]GABA.

Oxidation of [U-^{14}C]*glucose in cerebral-cortex slices.* The respiration rate of cerebral-cortex slices incubated in [U-^{14}C]glucose–saline medium was constant during the 40min experimental period, but the respiratory $^{14}CO_2$ production increased with time (carbon dioxide/glucose specific-radioactivity ratio 0.075 at 0–5min and 0.26 at 30–40min). These observations are similar to those reported by Beloff-Chain *et al.* (1955) and Cremer (1967) and indicate that appreciable time is required until the labelled intermediates equilibrate with the metabolite pools in the tissue. This view was supported by the observation that the specific radioactivity of alanine was only about one-third of that of glucose at 40min (Table 4).

The pattern of incorporation of glucose carbon into the amino acids in brain slices is consistent with the oxidation of glucose via glycolysis in the tricarboxylic acid cycle (Table 4). The highest incorporation rate was observed in alanine and the specific radioactivity of the amino acids decreased in the following order: glutamate, aspartate and GABA, glutamine. It should be noted that the specific radioactivity of glutamine relative to glutamate was about 0.5 during the whole experimental period, whereas that of aspartate and GABA

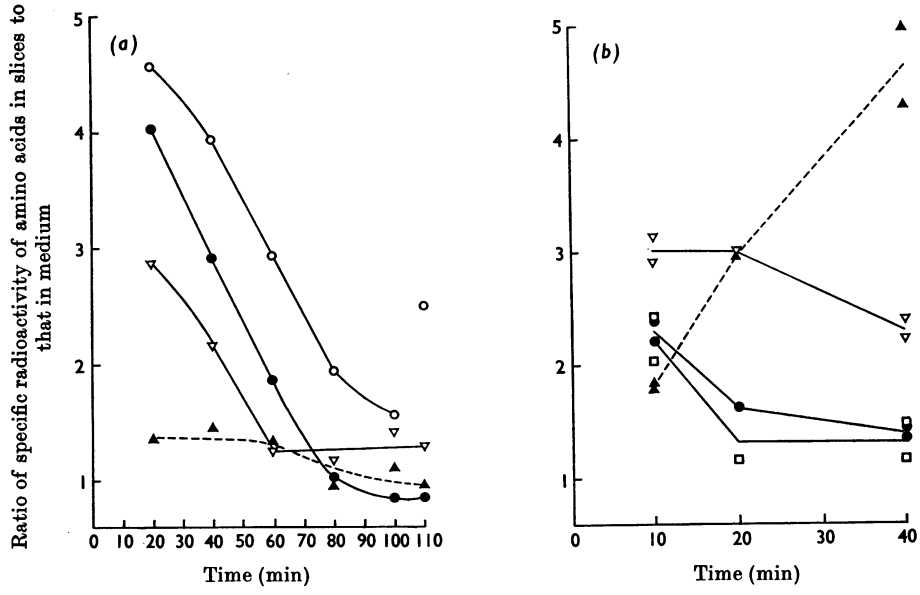


Fig. 4. Ratios of specific radioactivities of amino acids in slices of guinea-pig cerebral cortex to that in the incubation medium. (*a*) Brain-cortex slices were incubated in glucose–saline medium and the labelled substrate was [1-^{14}C]GABA. The data for calculation of the specific radioactivity ratios were taken from the results described in Tables 1 and 2 and Fig. 1. ○, Aspartate; ●, alanine; ▽, glutamine and ▲, glutamate. (*b*) In these experiments [U-^{14}C]glucose was the labelled substrate; the data were taken from the results given in Table 4. □, Serine; ●, alanine; ▽, glutamine; ▲, glutamate (the specific radioactivity of aspartate in the medium could not be determined in the experiments given in Table 4 because the ^{14}C content of this amino acid was too low).

respectively increased from about 0.5 at 10min to 0.8 at 40min. It has been shown that the glutamine/glutamate specific-radioactivity ratio was high when GABA was the labelled substrate (Fig. 1). Since both the labelled glucose and GABA are oxidized in the tricarboxylic acid cycle the results indicate that the glutamate pool is heterogeneous and the ^{14}C from these two precursors enters different glutamate pools.

The specific radioactivity of GABA was similar to that of aspartate during the whole incubation time. Isotopic exchange due to aminotransferase activity may be responsible for the labelling of glutamate and aspartate, but the conversion of [^{14}C]glutamate into [^{14}C]GABA is by metabolic incorporation of the radioisotope as a result of the involvement of irreversible steps in the formation of GABA (Haslam & Krebs, 1963; Balázs & Haslam, 1965).

The incorporation of ^{14}C into serine at 10min was the highest among the amino acids with the exception of alanine; thus glycolytic intermediates are involved in the biosynthesis of serine as in other tissues (Meister, 1965). Incorporation of ^{14}C into glycine, on the other hand, was not detected; thus the conversion of serine into glycine was negligible under the present conditions. Very little ^{14}C was detected in the region of the chromatograms that corresponded to GSH and its derivatives.

The content and distribution of amino acids in both slice and medium were similar to those described in the previous experiments with [1-^{14}C]-GABA as the marker. The increase in the ^{14}C content of alanine, glutamate and serine in the medium, although their concentration there was fairly constant, indicates that exchange took place with the amino acids in the tissue. The ratios of the specific activities of amino acids in tissue to those in the medium changed with incubation time (Fig. 4*b*). In Fig. 4(*a*) these ratios were plotted for the experiments in which [1-^{14}C]GABA was the labelled precursor. The comparison of the two sets of curves (Figs. 4*a* and 4*b*) indicate a marked difference in the equilibration of [^{14}C]glutamate between tissue and medium. The ratio of the specific radioactivity of glutamate in the tissue to that in the medium was already near to 1 at the initial period of incubation when [^{14}C]GABA was the precursor, but the ratio increased with time with [^{14}C]glucose as substrate. These observations

Table 4. *Incorporation of ^{14}C into amino acids of guinea-pig cerebral-cortex slices; incubation in [U-^{14}C]glucose–saline medium*

Slices of guinea-pig cerebral cortex (200mg wet wt.) were preincubated in glucose–saline medium for 10min, then 5μCi of [U-^{14}C]glucose (1.08×10^6 c.p.m.) was added (final vol. 3.0ml); gas phase O_2, temp. 37.5°C. The specific radioactivity (S.A.) is expressed in c.p.m./μg-atom of C; the S.A. of glucose in the incubation medium was 6200. The amounts of amino acids are expressed in μmol/200mg of slice and μmol/3ml of incubation medium respectively.

Time of incubation (min)	Alanine			Glutamate			Aspartate*			GABA†		Glutamine			Serine			Glycine‡	
	μmol in		S.A.	μmol in		S.A.	μmol in		S.A.	μmol in	S.A.	μmol in		S.A.	μmol in		S.A.	μmol in	
	slice	medium	(slice)	slice	medium	(slice)	slice	medium	(slice)	slice	(slice)	slice	medium	(slice)	slice	medium	(slice)	slice	medium
10	0.21	0.15	1060	2.06	0.10	155	0.48	0.08	90	0.47	90	0.67	0.53	84	0.25	0.13	237	0.31	0.14
	0.22	0.14	893	2.07	0.10	151	0.46	0.07	87	0.45	82	0.63	0.58	88	0.25	0.12	204	0.33	0.10
20	0.25	0.13	1697	2.05	0.11	369	0.46	0.07	237	0.42	229	0.67	0.76	195	0.28	0.12	340	0.35	0.15
40	0.27	0.14	2228	2.07	0.13	711	0.35	0.07	528	0.36	571	0.72	0.91	336	0.36	0.13	515	0.38	0.12
	0.24	0.13	2206	1.97	0.13	711	0.37	0.07	534	0.36	526	0.68	0.94	333	0.31	0.12	519	0.39	0.09

* The radioactivity combined in aspartate of the medium was too low for accurate determination.
† GABA was not detected in the medium.
‡ Radioactivity could not be detected in glycine either in the slices or in the medium.

Table 5. *Distribution of amino acids between slices of cerebral cortex and incubation medium, and between brain and cerebrospinal fluid* in vivo

The results *in vitro* used in the calculations were obtained in the present studies. The amounts of amino acids were expressed per g fresh wt. of brains (no correction for swelling was applied) and those in the medium were calculated as concentrations (μmol/ml). The amino acid contents *in vivo* were taken from the following references: (1) Mandel & Mark (1965); (2) Godin, Mark & Mandel (1968); (3) Perry & Jones (1961); and (4) Dickinson & Hamilton (1966). For the calculation of the ratios of concentrations of amino acids in tissue to those in the medium or cerebrospinal fluid (CSF), the tissue dry wt. was assumed to be 20% of the fresh wt.:

$$\text{Concn. ratio} = \frac{\text{concn. of amino acid in tissue fluid } (\mu\text{mol/g})\times(100/80)}{\text{concn. of amino acid in medium or CSF } (\mu\text{mol/ml})}$$

		In vitro						*In vivo*		
		(*a*) Rat		(*b*) Guinea pig				Brain	CSF	Concn. ratio
	Incubation time (min) ...	125		30		120				
	Amino acids	(μmol/g)	Concn. ratio	(μmol/g)	Concn. ratio	(μmol/g)	Concn. ratio	(μmol/g)	(nmol/ml)	
I	GABA	1.65	*	2.29	*	1.65	*	1.49[2], 1.89[1]	*	*
II	Glutamate	6.05	97	10.2	341	7.83	220	9.87[2], 10.1[1]	7[4], 7.5[3]	1600–1800
	Aspartate	1.60	56	2.30	129	1.53	110	2.75[1], 3.0[2]	1[4], 31[3]	100–3800
III	Glycine	1.48	40	1.73	43	2.07	30	1.20[1]	7[4], 15[3]	100–210
	Alanine	1.55	31	1.25	27	1.74	35	0.42[1]	23[4], 32[3]	16–23
	Serine	1.37	23	1.41	44	2.19	36	0.56[1]	38[4], 51[3]†	14–18
IV	Glutamine	3.98	15	3.37	16	3.37	11	3.0[1] 4.58[2]	220[3], 510[4]	7–26

* The concn. of GABA both in the incubation medium and in the CSF is too low for accurate determination; thus the concn. ratio for GABA is very high.
† The serine sample also contained asparagine.

suggest that the two substrates label glutamate in different compartments in the tissue and that the permeability properties of these compartments are not the same.

Metabolism of [U-^{14}C]*glucose in rat brain cortex slices.* This was also studied. The balance sheet at the end of 1h incubation in [U-^{14}C]glucose–saline medium was similar to that reported previously (see Beloff-Chain *et al.* 1955). About 20% of the glucose carbon utilized was converted into respiratory carbon dioxide, more than 60% into carboxylic acids and approx. 10% into amino acids. Although [^{14}C]glucose was present in excess, glucose did not accumulate in the tissue; thus the uptake of glucose kept pace with the metabolic requirements. Incorporation of ^{14}C was observed in neutral compounds, such as inositol, as also observed *in vivo* (Gaitonde *et al.* 1964). The medium, probably as a result of high concentration of lactate, contained about 90% of the ^{14}C combined in carboxylic acids. On the other hand, approx. 80% of the labelled amino acids were contained in the tissue. The pattern of ^{14}C incorporation into amino acids in the rat brain was similar to that observed with cortex slices from guinea pig. The specific radioactivities in decreasing order were as follows: alanine, glutamate, aspartate, GABA, glutamine. Incorporation was observed into serine, but the ^{14}C content of glycine was negligible.

Comparison of the concentration ratios of amino acids in vivo *and* in vitro. The concentrations of some amino acids in cerebral-cortex slices after incubation were different from the values determined *in vivo* (Table 5). The difference was related to the incubation time. The concentrations of glutamate and aspartate, in cortex slices from both guinea pig and rat, were near to the values *in vivo* for up to 1h of incubation. After longer incubation times, however, the concentrations of both amino acids decreased (Table 5). On the other hand, the concentrations of other amino acids, such as serine, glycine and alanine, were higher in incubated slices than *in vivo*. The content of GABA, which was elevated at the beginning of the incubation as a result of post-mortem changes, decreased during incubation of slices of guinea-pig cerebral cortex and approached the value *in vivo* (Fig. 2).

Amino acids were released from the tissue into the medium to a limited extent at the beginning of the incubation, after which the high concentration of amino acids in the slices was maintained against a considerable concentration gradient. The concentration ratios between tissue and incubation fluid were of four different magnitudes (Table 5): the retention of GABA by the tissue was the highest and retention of glutamine the lowest among the amino acids studied; the concentration ratios for the acidic amino acids were higher than for the neutral. The ratios observed *in vitro* are apparently similar to the ratios *in vivo* of the concentration in brain to that in the cerebrospinal fluid (Table 5).

DISCUSSION

Metabolism of GABA: previous estimates of flux

Using a complex model of the metabolic pathways in the brain, Garfinkel (1966) gave a tentative estimate of the flux in the GABA pathway as 20 or 34% of that through the tricarboxylic acid cycle. However, the experimental data he used in the computation probably overestimated the incorporation of ^{14}C from glucose into GABA (Cremer, 1964; Gaitonde *et al.* 1965), since the high specific radioactivity of GABA relative to glutamate obtained by Cremer (1964) was probably an artifact due to an unequal loss of ^{14}C from glutamate and GABA in the ninhydrin reaction before the counting of ^{14}C radioactivity. Further, Minard & Mushahwar (1966) have shown that the GABA/glutamate specific-radioactivity ratio increases in the brain after decapitation. The estimate of this ratio reported by Gaitonde *et al.* (1965) and Lindsay & Bachelard (1966) is therefore also higher than the true value *in vivo*, since in these experiments the metabolic reactions were not arrested immediately after death.

Other estimations of the GABA flux depended on measurement of the activities of the enzymes of the GABA bypath (Haslam & Krebs, 1963; Balázs & Haslam, 1965; Elliott *et al.* 1965; Myles & Wood, 1969). These experiments are open to the criticism that the utilization of an added substrate is not necessarily a valid indication of the rate of metabolism of intermediates generated in the cells. Thus it is known that there is a restricted entry of glutamate into subcellular structures. Another approach to estimation of the GABA flux was by the use of metabolic inhibitors, such as hydroxylamine (Haber, 1965) or amino-oxyacetic acid (Van Gelder, 1966). However, inhibitors are notorious in affecting a number of metabolic reactions simultaneously.

A value for the GABA flux of approx. 40% of that of the tricarboxylic acid cycle was obtained by McKhann *et al.* (1960) by incubating brain-cortex slices with [^{14}C]pyruvate as substrate and calculating the GABA flux from the specific radioactivites of glutamate and GABA. It was pointed out by these authors (Albers *et al.* 1961) that the calculations are not valid if glutamate is compartmented, but this high value for the flux through the GABA bypath is still quoted in the literature. The present study of the errors introduced by ignoring the compartmentation of glutamate in these experiments has shown that a large consistent overesti-

mation of GABA flux is to be expected under these conditions (Appendix, Hammond *et al.* 1970).

Evaluation of GABA flux: present studies

In the present work the GABA flux was estimated in brain-cortex slices prelabelled with [1-^{14}C]GABA and metabolizing unlabelled glucose. It was ascertained that after the initial uptake of [1-^{14}C]-GABA by the slice significant re-entry of ^{14}C into the labelled GABA pool did not occur either from the medium or from metabolites formed in the tissue (Tables 1–3). Evidence was also obtained that [^{14}C]GABA taken up by the tissue from the medium was contained in the endogenous GABA pool(s) (Machiyama *et al.* 1970), since on stimulation by K^+ the proportion of GABA released from the tissue was the same whether the slice contained exogenous GABA or not. Also the specific radioactivity of the liberated GABA approximated to that retained by the tissue whether GABA was labelled by exogenous [^{14}C]GABA or [^{14}C]GABA was generated in the tissue by metabolizing of [^{14}C]glucose.

The mathematical approach to the evaluation of the GABA flux under these conditions is given in the Appendix (Hammond *et al.* 1970). Considering first the results obtained on the assumption that GABA is metabolically homogeneous (Fig. 1*a* of the Appendix), the simulation of the decay of radioactivity combined in GABA gave the best agreement with experimental results when the fractional utilization rate constant of GABA (C_2) was 0.019/min (Fig. 5). The results for the time-course of GABA content were fitted by using this predetermined value for C_2. The solution that gave best agreement with GABA content is shown in Fig. 2. The corresponding value for the formation flux of GABA (K_1) is 0.0315 μmol/min per g. This value is 8% of the flux through the tricarboxylic acid cycle (0.401 μmol/min per g) calculated from the measured oxygen uptake of 72.8 μmol/h per g. The steady-state content of GABA was estimated to be 1.65 μmol/g, which agrees with the value obtained *in vivo* by extrapolation to zero time of the post-mortem changes of GABA content. The value obtained for GABA flux was also checked for consistency by computer simulation of the results for the radioactivity and the amount of aspartate (Fig. 6). The steady-state content of oxaloacetate was assumed to be 4 nmol/g (Goldberg, Passonneau & Lowry, 1966); the rate constants (per min) were as follows: oxaloacetate to aspartate, $C_4 = 63$; aspartate to oxaloacetate, $C_5 = 0.12$; oxaloacetate to citrate, $C_4^* = 100$.

The results of Machiyama *et al.* (1967, 1970) have indicated that GABA is compartmented in brain tissue. The effect of GABA compartmentation on

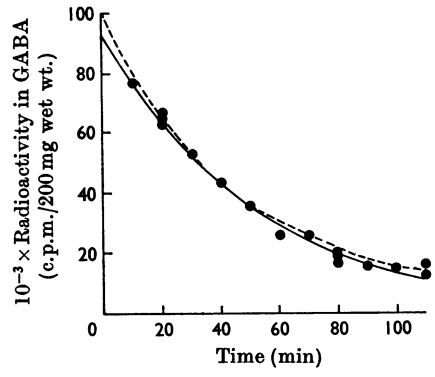


Fig. 5. The observed time-course of decay of [1-^{14}C]GABA in slices of guinea-pig cerebral cortex (see experimental details in Tables 1 and 2) was fitted on the basis of assuming (*a*) no compartmentation of GABA (——, representing computer solution based on Fig. 1*a* of the Appendix) and (*b*) two pools of GABA [– – –, described by the model in Fig. 1*c* of the Appendix and representing the equation $R = 40\,e^{-0.037t} + 60\,e^{-0.014t}$, where R is the content of ^{14}C in GABA (c.p.m.) and t is time in min]. The experimental points (●) were taken from Table 2.

the evaluation of the GABA flux was therefore considered (see the Appendix). The results showed that the values for the labelled GABA can also be matched on the basis of a model assuming two pools of GABA (Figs. 2 and 5). However, the mathematical postulation of compartmentation has little effect on the evaluation of the GABA flux, which was still 8–9% of the total flux through the tricarboxylic acid cycle.

Compartmentation indicated by GABA metabolism

The present results show that brain tissue *in vitro* retains to a considerable extent the metabolic and morphological organization underlying metabolic compartmentation (see also Berl, Nicklas & Clarke, 1968). Independent evidence was obtained that the pools of GABA and glutamate are both metabolically heterogeneous (Machiyama *et al.* 1970, and the present paper). Evidence of metabolic compartmentation *in vivo* and *in vitro* has also been obtained with other substrates (O'Neal & Koeppe, 1966; Berl *et al.* 1968; Van den Berg, Kržalic, Mela & Waelsch, 1969; Berl & Clarke, 1969). However, with GABA there are additional observations that help towards a better understanding of this phenomenon.

Compartmentation of GABA. It is relevant that GABA, and the enzymes directly associated with the formation and utilization of GABA, are present predominantly in grey matter (Roberts & Kuriyama 1968). The uptake of GABA *in vitro* also takes place

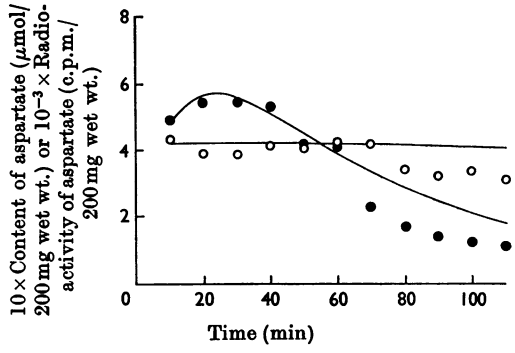


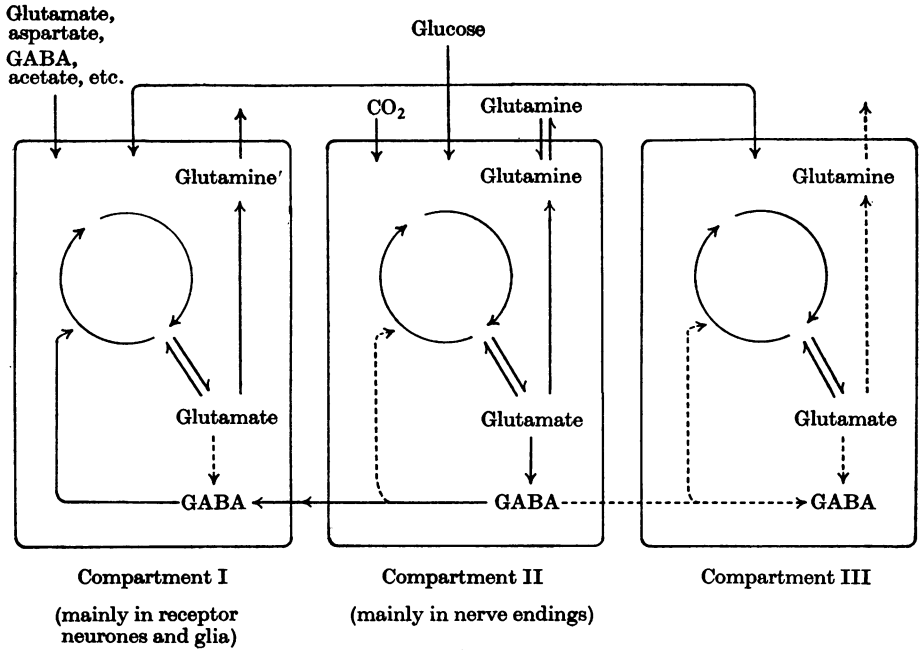
Fig. 6. Computer simulation of the time-course of the amount (○) and radioactivity (●) of aspartate. The labelled substrate was [1-^{14}C]GABA; experimental conditions were as described in Table 1. The mathematical approach is described in the Appendix (Hammond *et al.* 1970; model in Scheme 1*a*). Discrepancy between experimental points and computer curves (——) towards the end of incubation may be related to the decrease in the concentration of glutamate in the tissue at that time.

preferentially in grey matter (Nakamura & Nagayama, 1966). There is also evidence that GABA may be involved in transmission processes in the central nervous system (Curtis & Watkins, 1965; Krnjević & Schwartz, 1967). Further, evidence has been given elsewhere that a fixed proportion of the GABA behaves like transmitter compounds such as acetylcholine and the catecholamines and not like other amino acids in being released when brain-cortex slices are stimulated by depolarizing concentrations of K^+ ions (Machiyama *et al.* 1967, 1970). These observations suggest that the metabolism of GABA is somehow associated with nerve cells. However, it has been shown that not all types of nerve cells are equipped with the two main enzymes that produce and metabolize GABA: on the other hand one or both of these enzymes have been detected also in structures other than neurones, such as ependymal cells, blood vessels and the white matter of the cerebellum (Roberts & Kuriyama, 1968).

One of the GABA pools is associated with nerve terminals (Machiyama *et al.* 1970). In agreement with this view is the scheme of Salganicoff & De Robertis (1965) for the organization of GABA metabolism in the synaptic region: this is based on the observation that the synaptosomal fraction is relatively enriched in glutamate decarboxylase activity, whereas GABA aminotransferase is localized mainly in subcellular fractions containing non-synaptosomal mitochondria (see also Van Kempen, Van den Berg, Van der Helm & Veldstra, 1965; Balázs, Dahl & Harwood, 1966; Roberts & Kuriyama, 1968). GABA is therefore formed predominantly in nerve terminals, where it is also stored. In attempting to define the localization of the second GABA pool it is clear that the uptake and subsequent utilization of GABA cannot take place uniformly in all structures other than the nerve terminals, since the oxidation of GABA shows a compartmentation of glutamate. If it is taken that the liberation of GABA during transmission occurs near to the postsynaptic membrane of the adjacent neurone, that would imply that the second GABA pool is in the receptor neurones, although the involvement of the adjacent glial processes must also be considered.

Compartmentation of glutamate. This was shown by the following observations. The glutamine/glutamate specific-radioactivity ratio was greater than unity when the labelled substrate was [1-^{14}C]-GABA (Fig. 1); thus the intermediates formed from GABA had preferential access to a part of the total glutamate. When [U-^{14}C]glucose was oxidized this ratio was low; hence glucose had access to all or the major part of the glutamate (Table 4). The permeability properties of the compartments containing amino acids labelled by [^{14}C]GABA and [^{14}C]glucose respectively were different (see results for glutamate in Fig. 4); and stimulation by K^+ affected exclusively those compartments that became labelled through the oxidation of [^{14}C]-GABA (Fig. 7 of Machiyama *et al.* 1970). These results imply that the enzymes of the tricarboxylic acid cycle are also compartmented (see also Van den Berg *et al.* 1969), since ^{14}C can only enter glutamate and the other amino acids from [^{14}C]GABA by passing through the reactions of the cycle.

Scheme of metabolic compartmentation (*Scheme* 2). The functional compartmentation independently shown by GABA and by glutamate may be unified into a single model. On the basis of the compartmentation of GABA described above, one pool of glutamate is associated with the formation of the synaptic pool of GABA (compartment II). Another pool of glutamate must be associated with that compartment in which the main utilization of GABA occurs. This pool cannot be localized in the nerve endings because of the very low concentrations of GABA aminotransferase in this structure. The volume ascribed to the nerve endings has been estimated to be at most 15% of the total volume of brain (Salganicoff & Koeppe, 1968), and since [^{14}C]GABA utilization leads to the labelling of only a small fraction of the total glutamate, it follows that glutamate associated with GABA oxidation cannot represent all the glutamate in the remaining 85% of the brain volume. Hence, there must be more than one pool of glutamate besides that in the nerve terminals. One of these pools is directly linked with the oxidation of GABA, and may be situated on the basis of the allocation of $GABA_{(I)}$ in



Scheme 2. Simplified scheme of metabolic compartmentation in brain tissue. The compartments are defined on a functional basis, mainly considering the metabolism of [^{14}C]GABA. Compartment I appears to be associated predominantly with postsynaptic receptor neurones and also in part with the glial cells. Compartment II is associated with the nerve endings. Compartment III is not clearly localized. The flux through the tricarboxylic acid cycle is relatively high in compartment II and low in compartment I. Dashed lines indicate probably relatively low reaction rates. Communication between compartments is possible, e.g. through glutamine. The compartments to which exogenous substrates have access are indicated. Glucose enters all compartments freely. Substrates that elicit metabolic compartmentation are not necessarily all utilized at the same sites. The combination of pools constituting what is defined on a functional basis as a 'small' or a 'large' pool will depend on the substrate used, e.g., the 'small' glutamate pool for GABA, acetate etc. is probably associated with compartment I; hence the 'large' pool is the sum of compartments II and III. On the other hand, $^{14}CO_2$ in the presence of elevated concentrations of ammonia probably labels the 'small' glutamate pool in compartment II; hence the 'large' pool is then the sum of compartments I and III.

the postsynaptic part of the receptor neurones and probably also in part in some glial cells (compartment I). The third glutamate pool cannot yet be clearly defined in morphological terms: it represents the sum of compartments in which the fate of GABA is different from that in either compartment I or II.

The model given in Scheme 2 would explain a number of observations related to the metabolic heterogeneity of glutamate. The utilization of substrates such as glutamate, aspartate and acetate results in a high specific radioactivity of glutamine but in low specific radioactivity of GABA compared with glutamate. These observations indicate that GABA is preferentially formed from a pool of glutamate other than the 'small' pool associated with the metabolism of these substrates. However, it is also unlikely that GABA would be formed from the 'large' pools of glutamate associated with the main part of [^{14}C]glucose metabolism. The flux through the GABA bypath was estimated from the present experiments to be less than 10% of that through the cycle; nevertheless the incorporation of ^{14}C into GABA from [^{14}C]glucose was as high as into aspartate (Table 4). Further, the results of Minard & Mushahwar (1966) have indicated that the precursor of the GABA formed *post mortem* is a glutamate pool whose specific radioactivity as a result of [^{14}C]glucose metabolism exceeds the mean value. These observations are consistent with the entry of 'compartmentation' substrates into compartment I, whereas the rate of GABA formation is relatively high in compartment II, which is also characterized by relatively rapid oxidation of [^{14}C]glucose in a tricarboxylic acid cycle associated with a small glutamate pool. It may be noted that the subcellular distribution of some of the enzymes associated with the metabolism of 'compartmentation' substrates, such as glutamate dehydrogenase

and acetate thiokinase, coincides with that of GABA aminotransferase (compartment I) (Neidle, Van den Berg & Grynbaum, 1969).

Compartmentation of glutamate is manifested with $^{14}CO_2$ as the precursor under certain conditions (Berl & Clarke, 1969). The enzymes involved in carbon dioxide fixation are localized predominantly in the synaptosomal fraction (compartment II), whereas the enzymes critical in the utilization of the other 'compartmentation' substrates are found mainly in the 'free' mitochondria (compartment I) (Salganicoff & Koeppe, 1968). These observations can be explained by considering that the combination of the glutamate pools constituting what is defined on a functional basis as a 'large' or 'small' pool will vary according to the site of metabolism of the substrate utilized.

Relative pool sizes. Some indication of the relative pool sizes of glutamine, glutamate and aspartate may be obtained by considering the time-course of their specific radioactivities when [1-^{14}C]GABA is metabolized (Fig. 1). If a simple relation is assumed between precursors and products of the kind $A \rightarrow B \rightarrow C$, the curve for the specific radioactivity of the precursor should intercept that for the product at its peak. The specific radioactivities of the precursors (aspartate and glutamate respectively) were markedly different from those of the products (glutamate and glutamine respectively) at their peaks. One explanation is that the effective amount of glutamate associated with the flow of ^{14}C was less than the measured values. The calculations indicated that the pool of glutamate that was the precursor of glutamine was about 30%, and the pool that was the product of aspartate was about 50%, of the total glutamate in the compartment in which aspartate became labelled. The results given in Fig. 1 also suggest that the equilibration of the glutamate pools, either directly or via glutamine, is relatively slow: the peak of the specific radioactivity of glutamate is reached at 10min after that of aspartate, but the maximum specific activity of glutamine is at 40min after that of glutamate.

The results of Machiyama *et al.* (1970) may be used for the estimation of the sizes of the GABA pools. Since a fixed proportion of GABA is released on stimulation by K^+, compartment II (nerve endings) should contain approximately 25–30% of the total amount in the tissue.

Glial cells and compartment I. Part of the metabolism in compartment I is probably associated with the glia; this is suggested by the following considerations. (1) The axonal terminals and the adjacent neurones are enwrapped by glial processes: thus a part of the GABA released from presynaptic sites could be taken up by glia. (2) It is known that GABA aminotransferase is an ubiquitous enzyme; it is therefore likely to be present also in the less-specialized cells in the nervous system. (3) The metabolic pattern of compartment I indicates that a wider range of substrates (fatty acids and amino acids) is utilized for energy production than is characteristic of the brain as a whole (Van den Berg, 1970). This suggests a lower degree of specialization of the cells involved in the metabolism of compartment I than in the major functional units of the brain. This view is supported by the observation that, in contrast with neurones, glial cells can multiply even in the adult brain (Smart & Leblond, 1961). (4) The results of Van den Berg *et al.* (1969) and Nicklas, Clarke & Berl (1969) may be taken to indicate that the turnover rate of the tricarboxylic acid cycle is slower in compartment I than in the rest of the tissue. Neuronal function is associated with an active energy metabolism that would involve a relatively rapid flux through the tricarboxylic acid cycle. (5) Margolis, Heller & Moore (1968) found that the glial matrix remaining after the degeneration of the neurones in rabbit brain have a relatively low content of glutamate and a high content of glutamine. These observations support the view that the glia contribute to the processes assigned on functional grounds to compartment I. It may be possible to define the anatomical localization of the different compartments more precisely when the metabolic characteristics of the various different types of neurones and glial cells are better understood.

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APPENDIX

Mathematical Approaches to the Evaluation of the Flux of γ-Aminobutyrate in Brain Tissue *in vitro*

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In the preceding paper (Balázs, Machiyama, Hammond, Julian & Richter, 1970) the flux of γ-aminobutyrate (GABA) was found, in guinea-pig brain-cortex slices incubated in glucose–saline medium, to represent about 10% of the total tricarboxylic acid cycle flux, as opposed to other estimates, which are as high as 40%. However, the latter value was deduced from experimental results by methods that

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